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A solid-phase CuAAC strategy for the synthesis of PNA containing nucleobase surrogates

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Keywords: click chemistry, pyrene, fluorene, metal-binding, hairpin, on-resin, Huisgen cycloaddition

Abbreviations: CuAAC, copper-catalyzed azide-alkyne cycloaddition; DCM, dichloromethane; DIPEA, diisopropylethylamine; DMF, *N,N*-dimethylformamide; EDC, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide HCl; HOBt, hydroxybenzotriazole; HRMS, high resolution mass spectrometry; PG, protecting group; PNA, peptide nucleic acid; SSPS, solid phase peptide synthesis; THF, tetrahydrofuran

The synthesis of an azide containing PNA monomer is described. The monomer was incorporated into two PNA sequences for the purpose of synthesizing an intercalating fluorophore-labeled PNA and a metal binding hairpin using a solid phase copper catalyzed azide-alkyne Huisgen cycloaddition (CuAAC). Click chemistry was performed using 2-ethynylfluorene or 1-ethynylpyrene to add a fluorophore to the PNA, which were tested for their ability to recognize an abasic site on a DNA target. A PNA hairpin possessing azide monomers at each termini was synthesized and reacted with 2-ethynylpyridine to form a hairpin that is stabilized by Ni^{2+}.

Introduction

The synthesis of the first *N*-(2-aminoethyl)glycine-based peptide nucleic acid (PNA), a thymine homopolymer, was described in 1991 by Nielson and coworkers.[1] Following this landmark publication, in 1994 the details for the synthesis of the complete set of the PNA monomers of the four natural nucleobases and their oligomerization was disclosed.[2] Due to PNA's ability to bind strongly and selectively to complementary sequences, it has stimulated interest in a variety of applications from nucleic acid detection to supramolecular chemistry.[3] Concomitant with the high interest that PNA elicited, a variety of chemistries were developed for the synthesis of oligomers that spanned Boc, Fmoc and Mmt-based oligomerization strategies among others.[2,4-6] Our own interest in developing PNA chemistry was driven by the need for orthogonal protecting groups for the synthesis of nucleobase modified PNAs.[7,8]

PNA bearing modified nucleobases have been explored for endowing PNA oligomers with new biophysical properties, as a labeling strategy, or to incorporate expanded functionality such as fluorescence.[9] A straightforward strategy for making oligomers bearing modified nucleobases is via the preparation of the monomer and its incorporation during solid phase peptide synthesis, **Figure 1A**. This is a popular strategy, which we consider an early introduction of the modified base, but does suffer from the variable yields achieved for the coupling of the nucleobase submonomer to the protected backbone submonomer and subsequent inevitable losses of material during purification of the monomer ester and monomer acid.

An approach that avoids the tediousness of preparing individual monomers involves the use of a convertible or reactive monomer that can be transformed into a diversity of derivatives.[10] This approach is suitable at the monomer level (**Fig. 1B**) and may be performed on the oligomer while still attached to the solid support or post synthetically (**Fig. 1C**).[11,12] These two approaches represent a later stage of modified base introduction, as compared with approach A, and can be labor and material saving in their execution.

Of late, we have been interested in late stage introduction of nucleobase replacements for the aforementioned reasons. In the approach outlined below, **Figure 2**, the Huisgen copper-catalyzed azide alkyne cycloaddition (CuAAC) is used to make an integral modification[13] rather than a pendant one.[14]

Results and Discussion

PNA monomer synthesis. Many modifications to nucleobases are done as a pendant group; however, we were interested in a modification that was an integral structural component and the CuAAC was viewed as a way to achieve this.[13] Our original synthetic strategy toward PNA bearing nucleobase substitutions introduced by CuAAC "click" chemistry essentially followed the route illustrated in **Figure 1A**. As exploratory chemistry, we performed CuAAC with azidoacetic acid and some simple alkynes but found the products difficult to isolate, especially in the case

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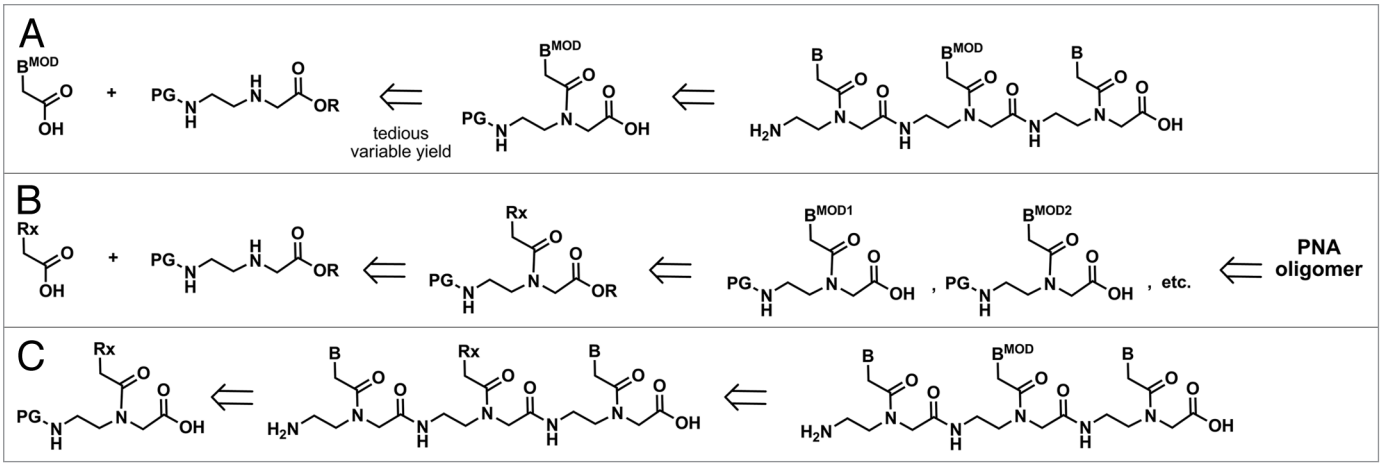


Figure 1. Illustration of the common retrosynthetic strategies for the synthesis of PNA oligomers bearing modified nucleobases. (**A**) Synthesis of individual monomers from their respective nucleobase acetic acid derivatives and a suitably protected backbone. (**B**) Synthesis of a common precursor monomer that may be chemoselectively transformed into various derivatives. (**C**) Incorporation of a convertible /reactive monomer into an oligomer and its subsequent transformation. BMOD, modified base; Rx, reactive group.

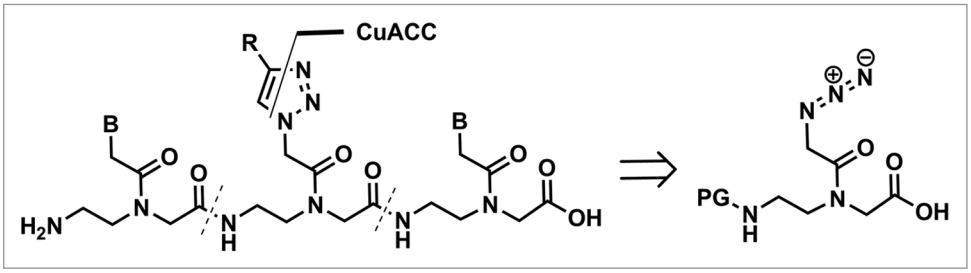
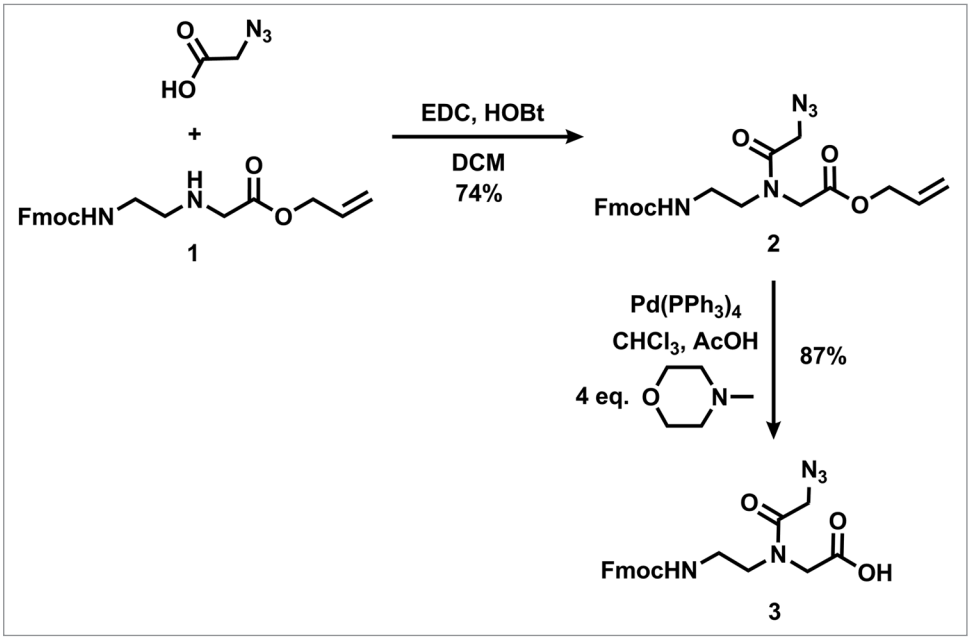


Figure 2. Retrosynthetic approach to base surrogates introduced by CuAAC requires an azido-containing monomer.



Scheme 1. Synthesis of azide-containing PNA monomer 3.

of 2-ethynylpyridine as the alkyne component. This led us to investigate the route shown in **Figure 1B**, wherein a PNA monomer capable of undergoing click chemistry was prepared (**Scheme 1**, monomer 2). While studying the model reaction with 2-ethynylpyridine we found that the monomer had low organic solubility after removal of the carboxyl protecting group. Finally, we settled on the approach illustrated in **Figure 1C**. We prepared a monomer suitable for Fmoc-based SPPS (3), as shown in **Scheme 1**, due to the popularity of Fmoc-based oligomerization and to permit cleavage and analysis of the azide-modified PNA; whereas the azide group has the potential to react (e.g., the Schmidt reaction) under the strongly acidic conditions required for cleavage of the PNA from the resin in standard Boc-based chemistry. We also planned to take advantage of the benefits of doing the CuAAC while the oligomer was bound to the solid support, such as using an excess of soluble reagents to favor products of reaction and the ability to wash out these reagents ultimately simplifying the purification. This resin-bound conversion of the oligomer is a technique that has been exploited by other groups.[15,16] This approach

also has the obvious benefit that many derivatized PNAs could be accessed from a single peptide synthesis by a partitioning the resin and carrying out several reactions in parallel.

The azide PNA monomer required a carboxylic acid protecting group that could be cleaved without using basic or reducing conditions, making the allyl ester an attractive choice (**Scheme 1**). The Fmoc/allyl protected PNA backbone[7] was coupled to azido acetic acid to produce 2 in 74% yield. Deallylation of 2 to reveal the carboxylic acid proceeded in 87% yield and provided the azide PNA monomer 3.

Abasic site detection. One application of nonnatural bases in nucleic acid analogs is to target sites of DNA damage, such as abasic sites.[17] A PNA containing an environmentally sensitive fluorophore could be used to give a fluorimetric response, indicating the location of the damage, as illustrated in **Figure 3**.[18]

The synthesis of fluorophore-containing PNA sequence was performed in a divergent fashion, where one sequence underwent a solid phase CuAAC with two different alkynes. A 7-mer PNA (PNA-Az) was synthesized with an internal azide residue and reacted with 2-ethynylfluorene or 1-ethynylpyrene to produce PNA-F and PNA-P, respectively (**Scheme 2**). The click reaction was performed in DMF or THF using alkyne, CuI, and DIPEA. After washing and cleavage from the resin, the crude PNA oligomer was analyzed using LC/MS, which showed complete consumption of the starting material. To calculate the concentration of the PNAs for subsequent hybrization studies, a model compound of the fluorene click product was synthesized (2-[4-(9H-fluoren-2-yl)-1H-1,2,3-triazol-1-yl]acetic acid, 4) (see **Supplemental Materials**). The ε(260 nm) value of *N*-methyl-1-aminopyrene was used for the pyrene-triazole insert.[19]

UV-thermal melt analysis was performed on duplexes formed between the 7-mers PNA-F and PNA-P and an 11-mer complement with a central abasic site (DNA-S, **Fig. 4**). The measured Tm values were lower than expected for PNA:DNA heteroduplex 7-mers (Tm ~36–43°C) in which the central residue was varied among all four possibilities. Estimation of the PNA:DNA duplex was based on the method described in reference 20. This calculation is based on the DNA:PNA duplex (3'-CAA-CGS-GAC-CT5: N-TGC-X-CTG-C (X=A: 42.1°C; G: 49.8°C; C: 43.3°C; T: 36.3°C). The corresponding DNA:DNA duplex (3'-CAA-CGS-GAC-CT-5', 5'-TGC-X-CTG-3') [X=A, G, C, T mixmer with all possible nucleobases across from abasic (S) site] displayed a monophasic transition yielding a Tm of 64°C at 2 μM strand concentration.[20] The oligomer containing the pyrene modification formed a duplex that was less stable than the complex formed by the oligomer containing the smaller fluorene modification, **Table 1**.

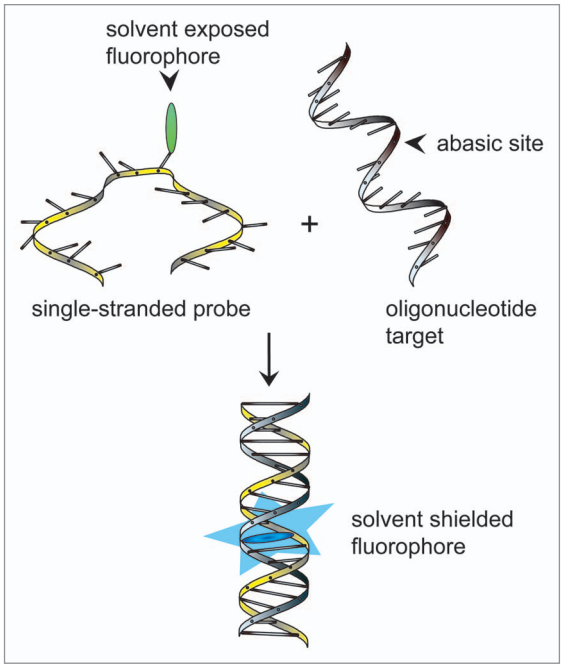
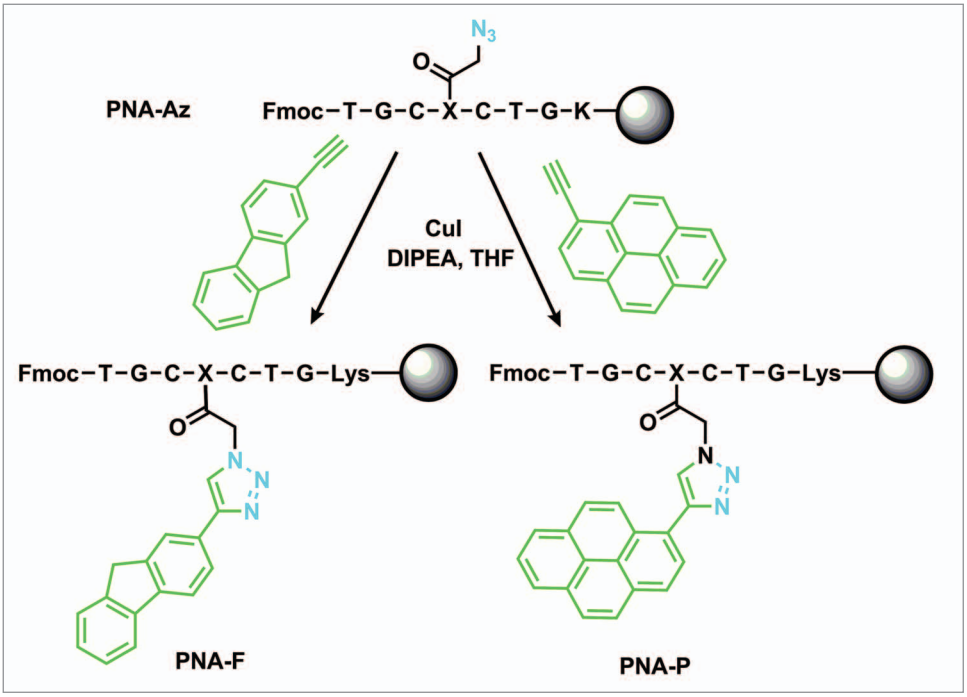


Figure 3. Idealized concept of an environmentally responsive fluorogenic base surrogate-based detection of an abasic site wherein a probe is nonemissive while exposed to solvent and demonstrates a change in emission when bound to the target.

These oligomers were expected to exhibit a change in fluorescence due to hybridization and concomitant intercalation of the fluorophore; however, neither did so. We interpret this combined



Scheme 2. Solid phase CuAAC synthesis of fluorophore labeled PNA.

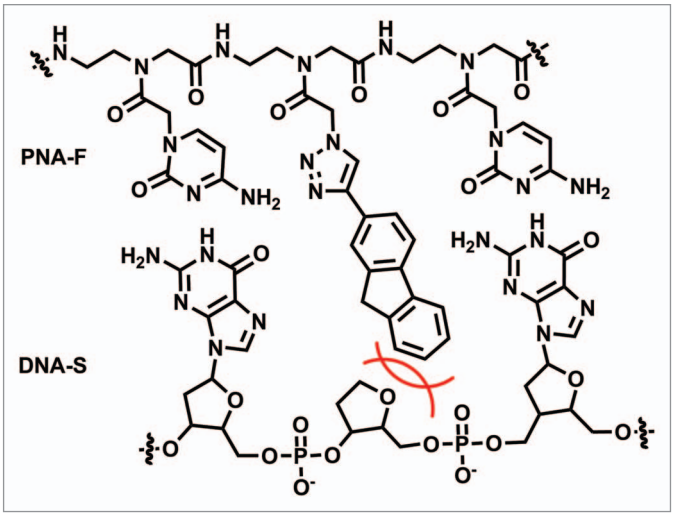
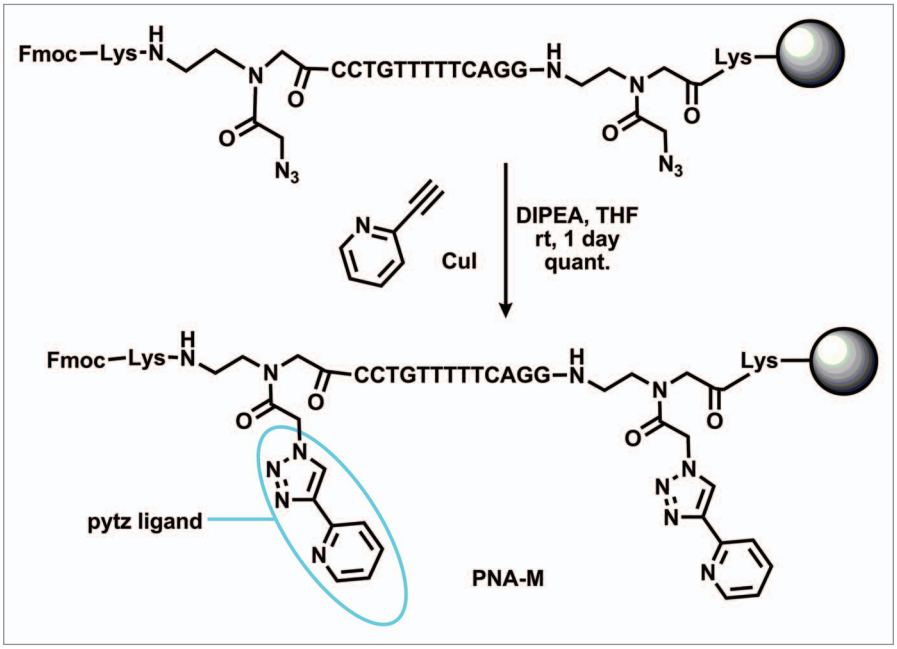


Figure 4. Proposed binding model for PNA-F to DNA containing an abasic site (DNA-S) illustrating the potential unfavorable steric interaction.

Table 1. PNA sequences and T_ms

	Sequence*	Tm (°C)§ DNA:PNA duplex
PNA-F	^{N}T-G-C- **F** -C-T-G-K^{C}	24.0
PNA-P	^{N}T-G-C- **P** -C-T-G-K^{C}	20.5

*F, fluorenyltriazole monomer; P, pyrenyltriazole monomer. §DNA target sequence: 3'C-A-A-C-G-S-G-A-C-C-T5', S, abasic site. Each strand present at a concentration of 2 μM in a buffer containing NaCl (100 mM), EDTA (0.1 mM), and Na_2PO_4 (10 mM, pH 7).



Scheme 3. Solid phase synthesis of PNA possessing metal chelating ligands as a product of the CuAAC.

behavior, a depressed Tm and lack of fluorimetric response, as a result of the fluorophore not being accommodated into the binding pocket provided by the abasic site (**Fig. 4**). A more compact fluorophore structure may be necessary for abasic site detection; however the current fluorophores may be useful as sequence end caps at the termini of PNA where there is more freedom from steric interactions and are being investigated as such.

Metal-binding PNA hairpin. Nucleic acids' ability to recognize complementary sequences has allowed them to carve out a niche in the molecular machines field.[21] With modifications, nucleic acids and analogs can act as molecular switches and logic gates, keeping them at the forefront of molecular computing.[22] A self-recognizing PNA whose conformation is dependent on the concentration of an ion has the potential to act as a molecular switch. As well, metal-mediated base pairing is of current interest in nucleic acid chemistry,[23] and metal chelation can be used to yield improved binding specificity of sequence probes.[24]

A PNA hairpin was designed to incorporate the azide monomer 3 on both ends, later to be transformed into a metal-binding ligand (**Scheme 3**). A click reaction was performed using 2-ethynylpyridine to form the 4-(2-pyridyl)-1,2,3 triazole (pytz) ligand which has been shown to bind to first row transition metals.[25] The concentration of PNA was determined using a model pytz compound (2-[4-(pyridin-2-yl)-1H-1,2,3-triazol-1-yl]acetic acid, 5) (see **Supplemental Materials**).

Melting studies found that the hairpin structure PNA-M was too stable to measure in water necessitating the use of urea as a chaotropic agent (Tm 57°C in 6 M urea). A small selection of metals that could accommodate square planar coordination were screened [Cu(II), Zn(II), Ni(II), Hg(II)] for their ability to stabilize the hairpin-structure based on increasing the Tm, under these conditions. The use of $HgCl_2$ showed an irreversible flattening effect of the melt curve, which we speculate involves the formation of duplex rendered completely self-complementary by the formation of Hg^{2+} bridged thymine-thymine bonds (see **Supplemental Materials**).[26] $NiCl_2$ was the only metal in our screen that showed stabilization of the hairpin structure.

The hairpin stabilizing ability was examined through a titration of the naked hairpin (4 μM) with increasing amounts of Ni^{2+}, **Figure 5**. The Tm, as determined by the first derivative method, showed a plateau of Tm = 68°C beyond 3 equivalents of Ni^{2+}, i.e. 12–20 μM show coincident melt curves. Further equivalents of metal ion changed the appearance of the cooperative melt curve without influencing the calculated Tm.

The reversibility of the metal stabilization was examined by the addition of EDTA and re-measuring the melting temperature (**Fig. 6**). The addition of EDTA reversed the Ni^{2+} stabilization back

to metal-free levels, but at high concentrations of Ni^{2+} and EDTA (320 μM) degradation of the PNA hairpin may have occurred because an erosion of the Tm was observed (see **Supplemental Materials**). DNA degradation has been described for Ni^{2+}·EDTA complexes[27] and it is conceivable that the implied metal chelate reactive oxygen species responsible for this could damage the PNA oligomer.

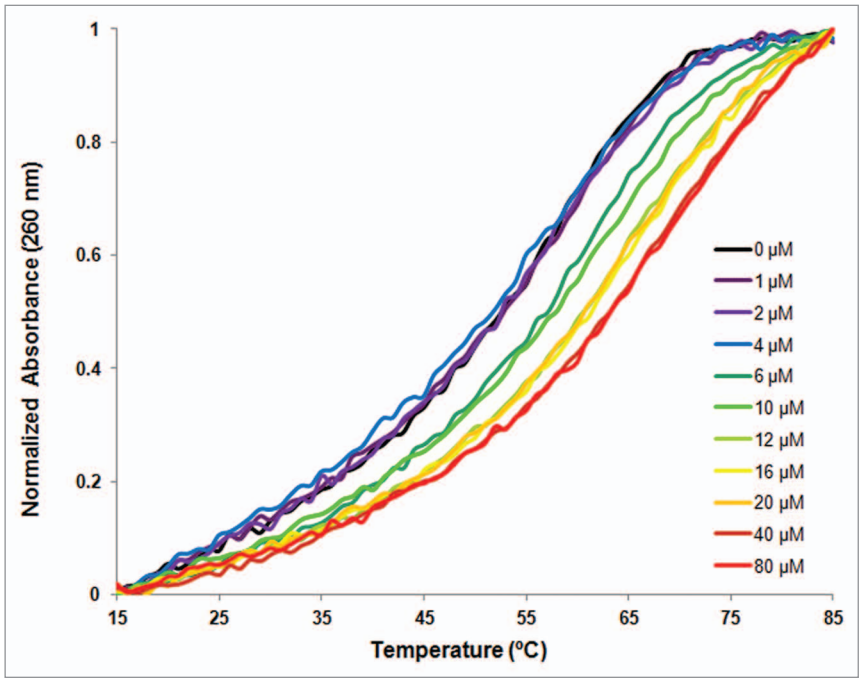


Figure 5. The effect of addition of $NiCl_2$ from 1 to 80 μM on the temperature-dependent absorbance at 260 nm. Hairpin concentration = 4 μM in 6 M urea.

Materials and Methods

General remarks. All chemicals were obtained from commercial sources and were of ACS reagent grade or higher and were used without further purification. Solvents for solution-phase chemistry were dried by passing through activated alumina columns. Flash column chromatography (FCC) was performed on Merck Kieselgel 60, 230–400 mesh. Thin layer chromatography (TLC) was performed on Merck Kieselgel 60 TLC plates. Chemical shifts are reported in parts per million (δ), were measured from tetramethylsilane (0 ppm) and are referenced to the solvent $CDCl_3$ (7.26 ppm), DMSO-*d6* (2.49 ppm), D_2O (4.79 ppm) for ^1H NMR and $CDCl_3$ (77.0 ppm) and DMSO-*d6* (39.5 ppm) for ^{13}C NMR. Multiplicities are described as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet) and br s (broad singlet). Coupling constants (*J*) are reported in Hertz (Hz). The ^{13}C NMR for 2 and 3 performed in $CDCl_3$ show the presence of rotamers. High resolution mass spectra (HRMS) were obtained using electron impact (EI) or electrospray ionization (ESI).

General procedure for solid phase click reaction. The resin was placed in a vial with either THF or DMF (1 mL) containing alkyne (5–50 eq.) and DIPEA (50–100 eq.). CuI (0.1–1 eq.) was added, the vial flushed with N_2, sealed and shaken overnight in the dark. The resin was added to a standard manual peptide synthesis vessel (internal volume ~5 mL) and the resin washed with DMF (3 mL), DCM (3 mL), MeOH (3 mL), H_2O (3 mL), MeOH (3 mL), DCM (3 mL) and then dried. The resin was returned to the peptide synthesizer for Fmoc deprotection using a standard protocol. The PNA was cleaved and purified following our previously reported procedures.[28]

PNA-Az. HRMS (ESI) calculated for $C_{76}H_{104}N_{40}O_{23}$: [M + 2H$^+$] 973.4177, found 973.9149; [M + 3H$^+$] 649.2811, found 649.6036.

PNA-F. HRMS (ESI) calculated for $C_{91}H_{115}N_{40}O_{23}$: [M + 2H$^+$] 1068.9608, found 1068.9182; [M + 3H$^+$] 712.9765, found 712.9348. Isolated yield: 20%.

PNA-P. HRMS (ESI) calculated for $C_{94}H_{114}N_{40}O_{23}$: [M + 2H$^+$] 1086.4569, found 1086.9707; [M + 3H$^+$] 724.6405, found 724.9683. Isolated yield: 20%.

PNA-M. HRMS (ESI) calculated for $C_{178}H_{230}N_{84}O_{50}$: [M + 4H$^+$] 1086.9588, found 1087.1980; [M + 5H$^+$] 896.7686, found 870.1615. Isolated yield: 30%.

2-Azidoacetate Fmoc/Allyl PNA monomer ester (2). A solution of azidoacetic acid (0.17 g, 1.7 mmol) and 1 (0.32 g, 0.85 mmol) were combined in DCM (5 mL) and cooled to 0°C. EDC (0.32 g, 1.7 mmol) and HOBt (0.038 g, 0.28 mmol) were added and the solution stirred for 10 min at 0°C, then an additional 18 h at room temperature. The reaction mixture was poured into DCM (100 mL) and washed with a saturated sodium bicarbonate solution (25 mL) and then brine (50 mL). The organic layer was dried over Na_2SO_4, filtered, and the filtrate was evaporated to dryness. The residue was purified by FCC (EtOAc-Hex = 3:2) to yield 2 (0.29 g, 74% yield) as a waxy white solid: ^1H NMR (400 MHz, DMSO-d_6) = 7.89 (d, *J* = 7.8 Hz, 2 H), 7.66 (d, *J* = 7.0 Hz, 2 H), 7.24 - 7.48 (m, 5 H), 5.84 - 5.98 (m, 1 H), 5.33 (d, *J* = 16.8 Hz, 1 H), 5.23 (t, *J* = 8.6 Hz, 1 H), 4.54 - 4.68 (m, 2 H), 4.25 - 4.38 (m, 2 H), 4.13 - 4.25 (m, 3 H), 4.09 (s, 1 H), 4.00 (s, 1 H), 3.30 (t, *J* = 6.4 Hz, 2 H), 3.07 - 3.24 (m, 2 H); ^{13}C NMR (101 MHz, $CDCl_3$) = 169.1, 168.5, 168.2, 156.4, 156.4, 143.6, 143.5, 141.0, 131.1, 130.7, 127.5, 127.4, 126.8, 124.8, 124.7, 119.7, 119.7, 119.4, 118.8, 66.6, 66.4, 66.3, 65.9, 50.1, 49.6, 48.4, 48.4, 47.9, 46.8, 38.8, 38.6; HRMS (ESI) calculated for $[C_{24}H_{25}N_5O_5]^+$: 464.1928, found 464.1923.

2-Azidoacetate Fmoc PNA monomer acid (3). Monomer 2 (0.291 g, 0.628 mmol) was dissolved in a mixture of chloroform (8.5 mL), AcOH (0.5 mL) and *N*-methylmorpholine (0.26 mL, 4 eq.) and degassed with N_2. $Pd(PPh_3)_4$ (0.01 g, 0.009 mmol) was added, the solution degassed thoroughly, and the solution stirred under an atmosphere of N_2 until complete reaction of the starting material (~7 d). The reaction mixture was poured into DCM (100 mL), washed with $KHSO_{4(aq)}$ (1 M, 25 mL) and the aqueous layer back-extracted with DCM (100 mL). The

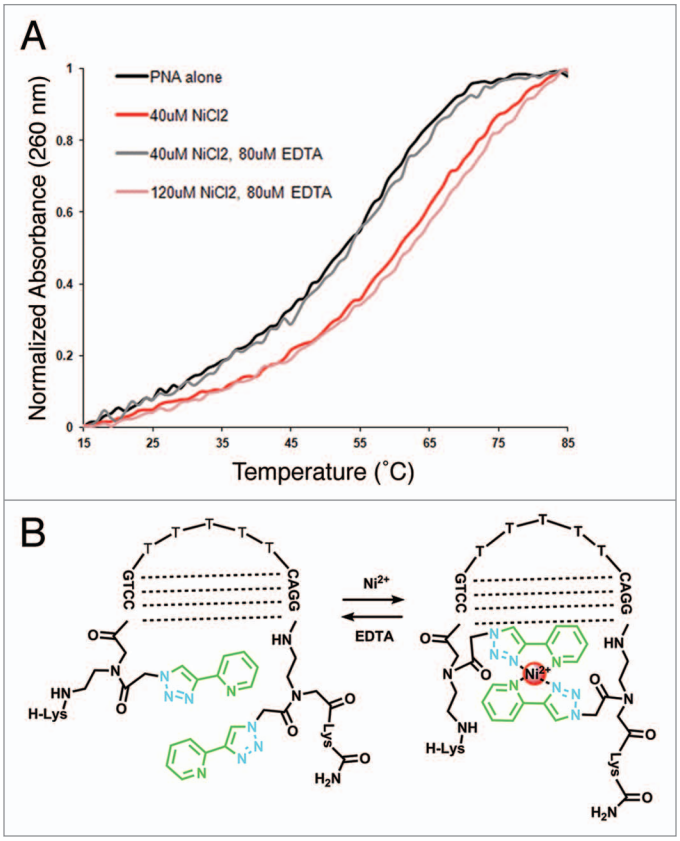


Figure 6. Left: the reversibility of metal-stabilization on the temperature dependent absorbance at λ = 260 nm. Hairpin strand concentration = 4 μM in 6 M urea. Right: cartoon of metal-based stabilization, and its reversibility, of the PNA hairpin with terminal 4-pyridyl-1,2,3,-triazole metal chelators.

combined organic layers were dried over Na_2SO_4 and evaporated to dryness. The residue was purified by FCC (EtOAc-AcOH = 99:1) to yield 3 (0.231 g, 87% yield) as a waxy white solid: ^{1}H NMR (400 MHz, DMSO-d_6) = 12.76 (br. s., 1 H), 7.89 (d, *J* = 7.4 Hz, 2 H), 7.62 - 7.71 (m, 2 H), 7.27 - 7.46 (m, 5 H), 4.26 - 4.38 (m, 2 H), 4.21 (t, *J* = 6.6 Hz, 1 H), 4.16 (s, 1 H), 4.05 (s, 1 H), 3.97 (d, *J* = 2.0 Hz, 2 H), 3.28 (t, *J* = 6.4 Hz, 2 H), 3.09 - 3.21 (m, 2 H); ^{13}C NMR (101 MHz, $CDCl_3$) = 172.2, 171.9, 171.0, 169.1, 168.2, 157.7, 156.8, 143.8, 143.6, 141.4, 141.2, 132.4, 132.2, 128.6, 127.9, 127.7, 127.4, 127.1, 125.1, 125.0, 124.7, 124.3, 119.9, 119.9, 67.1, 66.5, 50.4, 49.9, 49.7, 49.3, 48.8, 48.6, 47.1, 40.0, 39.6, 39.1; HRMS (ESI) calculated for $[C_{21}H_{22}N_5O_5]^+$: 424.1615, found 424.1621.

2-[4-(fluoren-2-yl)-1H-1,2,3-triazol-1-yl]acetic acid (4). 2-Ethynylfluorene (0.10 g, 0.53 mmol) and azidoacetic acid (0.20 g, 2.0 mmol) were dissolved in THF (5 mL). CuI (0.10 g, 1.3 mmol) and DIPEA (1.75 mL) were added and the solution stirred for 18 h. The solvent was removed, the residue suspended in $NaHSO_{4(aq)}$ (1 M, 100 mL), and extracted with Et_2O (2 × 100 mL) and EtOAc (2 × 100 mL). The combined organic layers were dried over Na_2SO_4 and evaporated to dryness. The residue was dissolved in boiling EtOAc (30 mL) and hot filtered. Hexane was added (60 mL) and the solution cooled on an ice bath. The solid was filtered to yield 4 (0.048 g, 31% yield) as a gray powder: ^{1}H NMR (400 MHz, DMSO-d_6) = 8.59 (s, 1 H), 8.09 (s, 1 H), 7.98 (d, *J* = 7.8 Hz, 1 H), 7.86 - 7.96 (m, 2 H), 7.61 (d, *J* = 7.4 Hz, 1 H), 7.40 (td, *J* = 1.0, 7.5 Hz, 1 H), 7.33 (td, *J* = 1.2, 7.4 Hz, 1 H), 5.32 (s, 2 H), 4.00 (s, 2 H); ε_{260} = 4870 ($A \cdot M^{-1} \cdot cm^{-1}$); HRMS (ESI) calculated for $[C_{17}H_{14}N_3O_2]^+$: 292.1086, found 292.1091.

Sodium 2-[4-(pyridin-2-yl)-1H-1,2,3-triazol-1-yl]-acetate (5). 2-Ethynylpyridine (0.33 g, 3.2 mmol) and azidoacetic acid (0.22 g, 2.2 mmol) were dissolved in THF (5 mL). CuI (0.025 g, 0.13 mmol) and DIPEA (1 mL) were added and the solution stirred for 2.5 h. The solution was diluted with THF (100 mL), filtered through Celite, and evaporated to dryness. The residue was dissolved in EtOH (10 mL) and that pH was adjusted to approximately 9 by use of 1 M NaOH in EtOH. The solid was filtered off and washed with EtOH (10 mL) to yield 5 (0.048 g, 31% yield) as a tan powder: ^{1}H NMR (400 MHz, DMSO-d_6) = 8.51 - 8.63 (m, 1 H), 8.34 (s, 1 H), 8.01 (dd, *J* = 0.8, 7.4 Hz, 1 H), 7.87 (td, *J* = 1.2, 7.8 Hz, 1 H), 7.23 - 7.37 (m, 1 H), 4.67 (s, 2 H); ε_{260} = 7040 ($AM^{-1}cm^{-1}$); HRMS (ESI) calculated for $[C_9H_9N_4O_2]^+$: 205.0726, found 205.0737.

Conclusions

In conclusion, an azide functionalized PNA monomer was synthesized and incorporated into PNA oligomers using Fmoc-based oligomerization protocols. On-resin CuAAC reactions were performed and proceeded in high yield. Oligomers containing single internal azide residue or dual labels at the termini were prepared and derivatized with hydrocarbon fluorophores (fluorene and pyrene) or pyridine, respectively. Divergent syntheses can be accessed by split-and-react process from the singly-labeled PNA.

The design of the azide-containing PNA monomer is such that the CuAAC produces a substituted triazole as a nucleobase replacement. In the case of reaction with 2-ethynylpyridine, the CuAAC results in the pyridyl-triazole ligand which can reversibly bind Ni^{2+} and form a metal-mediated base pair analog.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/artificialdna/article/23982

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